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BLOOD CELL REGENERATION IN LIMULUS POLYPHEMUS Part II

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BLOOD CELL REGENERATION IN *LIMULUS POLYPHEMUS*
Part II

A Major Qualifying Project Report:

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

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Approved:

Dr. Daniel Gibson III, Major Advisor

Abstract

This project continued the research of Hinkley and Lapointe (MQP, 2006). A key concern in horseshoe crab research is the survival rate of the crabs after they are bled for pharmaceutical purposes, i.e., endotoxin detection by amebocyte extracts. We designed a 6 week field study on the regeneration of blood cells in *Limulus polyphemus*. Our results indicate that surviving bled horseshoe crabs did rebound, showing an increase in blood cell proliferation that begins within days. A majority of the crabs (bled *and* unbled) demonstrated a rise in cell counts over initial values.

Acknowledgments

This project showed us a lot about the successes, failures, and uncertainties that can arise when performing field studies and laboratory science. Throughout this year much of what we learned can be attributed to the help of a few people. We would not have achieved the successes we have without their help and they deserve to be recognized.

Dr. Dan Gibson was an integral part of our project team and deserves as much credit for its completion as we do. The help you have given us in the setup and execution of this project was immeasurable. Thank you for all of your help.

Another person that also should be recognized is Mrs. Virginia Kuykendall of Upton, MA. Her permission to use her marshfront for our horseshoe crab pen was crucial. She wrote letters on our behalf to the town agencies and advised her summer tenants of our work. The summer of 2006 was the second year that she allowed us to use her property. Without her good will and interest, this project would not have been possible.

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Introduction

Each species that has lived on the planet has either evolved to the changing earth or become extinct. However, for the 300 million years that the horseshoe crab has existed, it has remained mostly unchanged. If the consistency of its body plan over time can be used to gauge the stability of its physiology, we can assume that the immune system of the horseshoe crab, *Limulus polyphemus*, is a truly ancient approach to prevention of infection and of blood loss.

Horseshoe crabs do not posses an immune system like the ones found in mammals; their blood cells, or amebocytes, have provided protection from disease and infection. The amebocytes attack and bind to the lipopolysaccharide type of bacterial endotoxin found in gram-negative bacteria and render it ineffective by forming a clot around it. Amebocytes thus act as a primitive immune system and also provide the mechanism for prevention of blood loss after injury. .

In the world of biotechnology today, there is a commercial product that is based on this natural phenomenon. This commercial product is called Limulus Amebocyte Lysate. LAL, as it is called for short, has the ability to signal the presence of bacterial endotoxins. Although LAL can actually facilitate the removal of endotoxins from contaminated drugs or devices, its normal use is only as a test for contamination, not as a removal system. The LAL system is a cascade of proteins that becomes active when any type of bacterial endotoxin is present in a system. The discovery of LAL and its cascade can be attributed to Frederick Bang in 1964 at the Marine Biological Laboratories at Woods Hole, Cape Cod. Dr. Bang was a Johns Hopkins researcher who was doing

research at the Woods Hole Laboratories when he found that when a bacterium was injected into a horseshoe crab, the blood became viscous due to the major clotting of the blood. It was later found that the amebocytes released granules that clotted around the bacterial endotoxins. This phenomenon was later developed into an assay in which LAL from horseshoe crabs can be used to detect any presence of endotoxin or bacteria that may occur in medical injectable solutions and the medical devices that are used to inject the solutions (http://www.horseshoecrab.org, 2007).

As the LAL industry continues to grow, the demand for horseshoe crabs is increasing. Along with the demand of horseshoe crabs for biotechnology purposes, there is also a high demand for crabs as bait for eel and conch fisherman. The eel and conch fisheries accounted for the death of roughly 2 million horseshoe crabs in 1996, (http://www.horseshoecrab.org,2007). Biotechnology companies such as the Associates of Cape Cod report that after the bleeding of the crabs, they are released back into the wild. However, they also report that there is roughly a 3% mortality rate in the crabs that they harvest. Due to the reported mortality rates the biotechnology industry is responsible for the deaths of roughly 20,000 to 37,500 crabs per year due to the bleeding process. There is concern over the sustainablility of horseshoe crab populations for bait and for biomedical uses. Horseshoe crabs mature very slowly and require 9 to 11 years to reach sexual maturity. Recruitment of breeding animals is thus very slow and could easily be outstripped by excessive harvesting.. Another major reason for the need of conservation is the fact that many eel and conch fisheries prefer using female horseshoe crabs that contain eggs. Removing breeding females is obviously a detriment to renewal of the population. The easiest time for fishermen (and lysate manufacturers) to harvest

horseshoe crabs is during the spawning season (locally May and June) when they come in to shallow waters. This means that they are easily exploited during their mating season. There is disagreement among the various groups that exploit horseshoe crabs as to how well they survive the bleeding process. Mortalities of 3-10% are assumed by most of the LAL manufacturers, while fishermen insist that a bled crab is as good as dead and may as well be used for bait. One of the aims of this study was to track mortalitly after exsanguination; another was to determine how fast blood cells are regenerated after bleeding.

Background

Horseshoe crabs belong to the Arthropod phylum, and have their own class called Merostomata, which means "mouth in the middle," which refers to the location of the mouth at the point where all the basal segments of the legs converge. They are more closely related to chelicerates like spiders and scorpions than to crabs and these “living fossils” are said to have existed in their present form more than 300 million years ago. *Limulus polyphemus* have a relatively simple anatomy which can be divided into three parts; the prosoma, the opisthosoma, and the telson. Within the prosoma lie the internal organs of the crab, the intestinal tract, the brain, heart, the eyes, and its walking legs. The opisthosoma contains the five pairs of book gills and the muscles used by the telson or the tail, which is the only part of the anatomy that grows disproportionately larger with each molt (Sekiguchi, 1988).

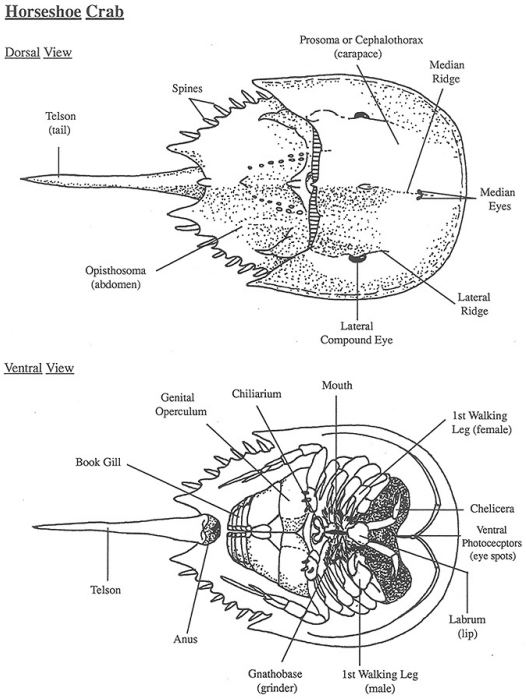

Figure 1.0 Basic Horseshoe Crab Anatomy (Copyright 1993, Maine Department of Marine Resources, Education Division)

The horseshoe crab has a relatively simple circulatory system. A tubular heart runs down the center of the body and along the entire length, just under the dorsal midline. The horseshoe crabs do not have a separate immune system as in vertebrates, but the circulating blood cells act as a primitive immune system as well as a clotting mechanism. In the 1960's it was discovered that when common bacteria from the "bacterial soup" of natural seawater were injected into the horseshoe crab, clotting occurred. This is because the blood cells known as amebocytes contain an active component referred to today as Limulus Amebocyte Lysate (LAL). The LAL is able to bind and inactivate the bacterial endotoxin and also forms a clot so that the crab stops bleeding to prevent infection in the crab, and can be used to detect endotoxin in biomedical products. The granulocytes or hemocytes of the blood, perform exocytosis as soon as they are able to detect the intruding gram-negative bacteria. This releases the clotting factors on the pathogens which results in the hemolymph coagulation seen in Figure 2.0 (Iwanaga, 1998). This phenomenon has kept marine biologists, chemists, and biomedical manufacturers busy for years.

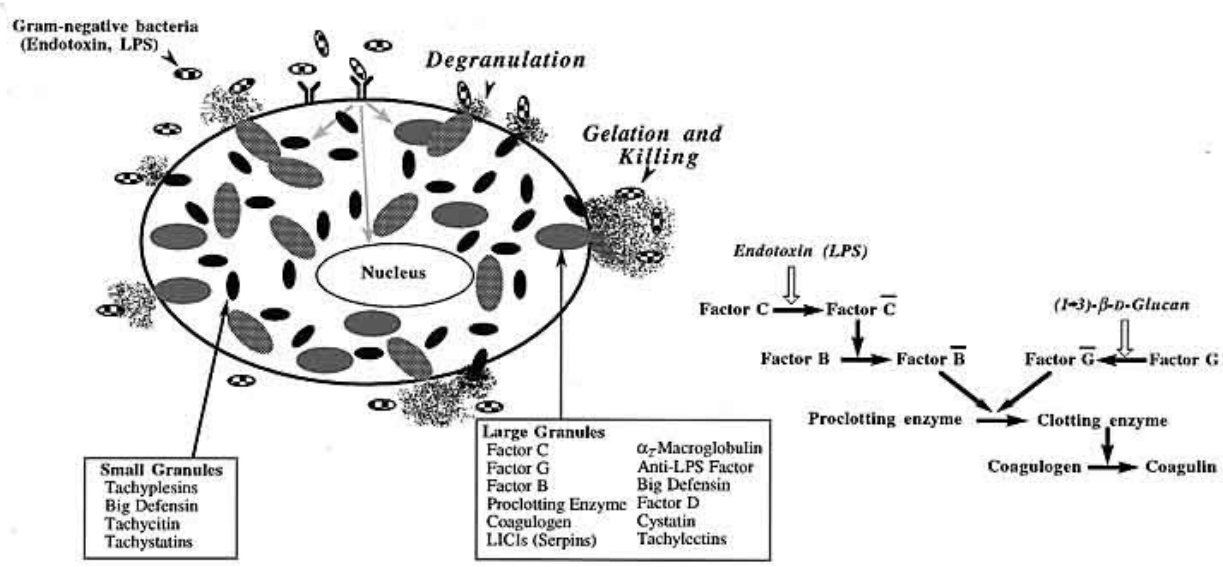

Figure 2.0 Defense Systems in Horseshoe Crab Hemocytes. (Iwanaga, 1998)

One of the first scientists to have any interest in the LAL was Dr. Stanley Watson of the Woods Hole Oceanographic Institution. Watson experimented with and produced the LAL reagent at a level small enough for his own personal use and for a few of his fellow scientists (http://www.horseshoecrab.org, 2007). Unfortunately the research was not kept secret for long and soon after the biopharmaceutical industry became extremely interested in the amazing power of the LAL; raising both the cost and demand. Dr. Stanley Watson would soon go on to start his own company known today as the Associates of Cape Cod (http://www.horseshoecrab.org, 2007).The Associates of Cape Cod was the first producer of LAL to receive their license from the Food and Drug Administration.

Today there are three simple procedures of LAL testing including the gel-clot test, turbidimetric test, and chromogenic test. The chief application for LAL is the testing of pharmaceutical products and medical devices that contact blood or cerebral spinal fluid such as dialysis machines, hypodermic needles, and other injected medications. Since 1973 when the FDA announced that LAL is a biological product, production techniques

have greatly improved (Center for Drug Evaluation and Research, 1987). The LAL test is recognized as an elite mode of detection for the presence of bacterial endotoxins and is now used as an industry standard (Center for Drug Evaluation and Research, 1987).

Literature Review

In the early 80's, marine biologist Anne Rudloe had completed several studies on the *Limulus polyphemus*. Although these animals are able to endure severe physical injuries and extreme levels of blood loss, no prior experiments had been done to show mortality rates of horseshoe crabs returned to their normal habitat after bleeding the animals for biomedical purposes. In 1981, Rudloe created a field experiment with a team of 22 volunteers to test the survival rates of released crabs, bled and unbled, compliant to industry standards.

Rudloe and her group began their experiment in St. Joseph Bay, Florida collecting a total of 10,259 adult horseshoe crabs; a sample size larger than any of its kind. Next, the crabs were to be divided into two groups. A total of 47% of the crabs were bled, while 53% represented the control. The bleeding process was simple enough to be preformed by a group of the 22 inexperienced volunteers. The crab was held in a support so that the muscle above the heart was exposed which was then swabbed down with 70% isopropyl alcohol, and a 16-gauge 1 ½ in. hypodermic needle was injected into the heart causing the blood to flow freely into a beaker. Once blood flow came to a slow drip the needle was removed and the blood volume was recorded.

Continuing with the experiment, each individual animal was tagged to indicate whether or not the crab had been bled so that recovery experiment could be possible, and was then released into the bay. Approximately two weeks later, Rudloe and her crew set out to recover the horseshoe crabs finding only a 28% maximum success rate of recovery. Of the 1415 crabs recovered, 85 of them were dead with a bled/control ratio of 55%:45%. In other words, out of the 85 dead crabs, 47 had been bled, while 38 were control. After many ratio tests and direct comparisons Rudloe's results show a 10% increase in mortality among the bled crabs over the rate of natural mortality. However, for the majority of the bled crabs they recover completely and are able to do so in a short time.

A similar experiment was done by Elizabeth Walls and Jim Berkson of Virginia Polytechnic Institute, who focused primarily on the determination of horseshoe crab mortality rates after the bleeding process in a controlled environment. In 1998 the Atlantic States Marine Fisheries Commission mandated that all biomedical companies responsible for the bleeding of horseshoe crabs must estimate the mortality rates resulting from the process. Walls and Berkson worked closely with one of the largest producers of LAL, BioWittaker, in response to the mandate by the ASMFC.

For their experiment, only on newly matured male horseshoe crabs were captured near Chincoteague, Virginia and Ocean City, Maryland. They were divided up into between bled and unbled groups at BioWittaker's bleeding facility in order to compare mortality rates. They focused solely on males in order to avoid variance within the experiment. Of the 200 crabs collected and bled, only 16 (8%) died versus the 1 (0.5%) of the control, revealing that horseshoe crab mortality due to bleeding is moderately low in a controlled environment. Although this cannot be directly correlated to the natural

habitat of the horseshoe crab, Elizabeth Walls and Jim Berkson estimated that approximately 18,750 horseshoe crabs die annually as a result of the biomedical bleeding process.

In the summer of 2001, W. Kurz and Mary Jane James-Pirri performed another experiment on the impact of the biomedical process on *Limulus polyphemus* in a small estuary on Cape Cod, Massachusetts. Again, the purpose of this experiment was to determine if the bleeding affects the horseshoe crab's survival rate and whether or not it has any influence on the crab's behavior. Although this experiment was relatively small with a total sample size of 20 crabs (10 bled/10 unbled), Kurz and James-Pirri focused solely on female horseshoe crabs. After following the crabs for 26 days, all of the crabs were recovered with the exception of 3; two bled and one control. No mortality was observed in the unbled group, while a mortality rate of 20% was observed within the bled group. However, the second phase of this experiment did show a significant difference in the behavior of the crabs. The horseshoe crabs that were bled appeared to move randomly throughout the estuary, while the control group showed a specific directional movement pattern. This data suggests that the experimental crabs experienced more disorientation post-bleed.

Materials and Methods

Our experiment involved a seven week study during the summer of 2006 from May 24th to July 5th. This study was a continuation of the previous 2006 study; BLOOD CELL REGENERATION IN *LIMULUS POLYPHEMUS, (Lapointe & Hinkley,2006).* The study began with the collection of 34 horseshoe crabs from 2 locations that make up the east and west sides of Phinney's Harbor in the Monument Beach area of Bourne, MA. The first collection site was near the town boat landing in Monument Beach, and the other was along Mashnee Dike, a sand barrier that separates Phinney's Harbor from the Cape Cod Canal. Only adult males were taken. Paired or breeding horseshoe crabs were not separated or removed from the population. Since the collection times coincided with the natural breeding times of horseshoe crabs, we wanted to have a minimal effect on breeding. The removal of only single adult males minimized covariance in the study; following the lead of Walls and Berkson (2000): "in an attempt to minimize variation of external influences, so that the only difference between the two groups was whether or not they underwent the blood extraction process." After all the crabs were collected they were then transported to the Great Sippewissett Marsh in West Falmouth, MA.

Building the Pen

The next stage of the experiment was the creation of a holding pen for the horseshoe crabs. The pen was created and kept at the Great Sippewissett Marsh in West Falmouth, MA, behind the barrier island of Chappaquoit Beach with the permission of the property owner, Mrs. Kuykendall. This holding pen would allow the crabs to remain in the most natural habitat possible for the duration of the study. Construction of the pen began with the pre-build layout of the PVC pipe in the shape of what would be the final

pen. It was built with 1 ¼ inch PVC pipe in a 10' by 10' square with 3' high walls. The posts of the pen were sunk into the sand at least 4 feet in order to keep the pen stable, and vinyl coated fencing with 2X3" mesh was attached to the posts so that it touched the sand bottom. To keep crabs from digging under the fencing, all sides of the pen were further lined with metal white foldable border fencing driven six inches below the sand surface to prevent escape by burrowing.. Each of the pieces of the fencing was attached to the PVC pipe with plastic zip-ties. This attachment method proved to be an excellent one because there were no holes in any of the areas of the pen that crabs could escape through.

Figure 3.0 PVC Pipe Pen

Full Bleed on Experimental Crabs

Once the crabs were introduced into their new home for the next six weeks, they were split into two separate groups. Half of the crabs were placed in the 'Experimental' group and the other half were placed into the 'Control' group. Each of the two groups was then marked with our marking system. The 'Experimental' group was marked with a colored thumbtack placed in the opisthoma above their left genal

spines. There are only 5 genal spines on each of the horseshoe crabs so different colored thumbtacks were used when the first 5 spines were used in marking. Different colored thumbtacks allowed us to have more than 5 crabs for each group. 'Control' crabs were identified by the different colored thumbtacks that were placed in the opisthoma above the right genal spines. The side of the crab, color of the tack and the number of the spine was used for the identification numbers for each crab, (LR5 = Left, Red, 5th spine).

When the 'Experimental' crabs were ready for the bleeding process, a 16 gauge needle was used to pierce the cartilage of the hinge, over the heart. After the needle was inserted into the heart, the blood would flow out of the needle and into a sterile beaker for collection. When blood flow slowed, we flexed the crabs several times at the hinge to express more blood. This procedure removed more blood than standard exsanguination during LAL manufacture. During the blood collection process, we estimate that 30% of the total blood volume was removed from each of the bled crabs. This process was repeated for each of the 11 crabs that remained in the 'Experimental' group.

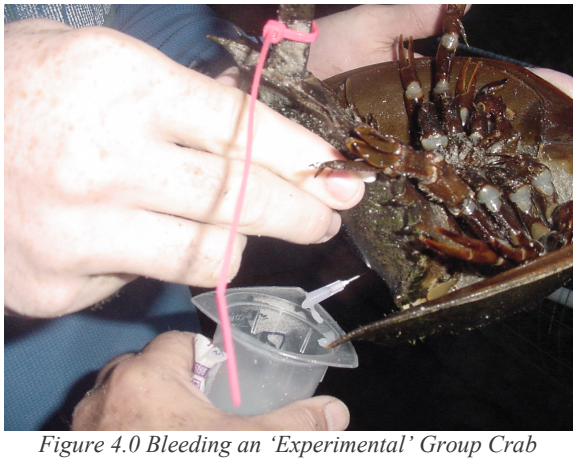

Figure 4.0 Bleeding an 'Experimental' Group Crab

Blood Sampling

The blood sampling process was the same for each of the groups used in the experiment. Blood sampled were drawn (cardiac puncture) into a solution of 1% paraformaldehyde (fixative), 3% NaCl (osmotic agent) and 0.5% Tween 80 (a detergent to prevent cells from clumping). The cardiac puncture process involved inserting a sterile 20 gauge syringe needle into the heart through a soft spot on the back of the horseshoe crab located in the center of its hinge that separates the opisthosoma and the prosoma. Each of the samples that were taken was made up of 0.5mL of blood from the horseshoe crab and 2mL of the solution creating a 1:5 solution. The samples were collected over the course of the remaining 6 weeks of the experiment. All of the samples were transported to the laboratories at WPI and placed in a refrigerator to be counted at a later date.

Figure 5.0 Blood Sampling Individual Horseshoe Crabs

Counting of the blood samples involved the use of a microscope, hemocytometer, tissue homogenizer, 20 μL pipetter and sterile pipette tips. The syringes

that contained the blood samples were poured into a tissue homogenizer and mixed so that there was no clotting of the blood cells in the sample. After the sample was homogenized, 10 µL of the blood was collected with the pipetter and then injected onto a hemocytometer to be counted. The cell counts for each of the crabs was counted by both members of the group and then the total cell numbers that was counted by the members of the group was then averaged together for each crab so that a more accurate count of cells/mL was achieved.

Figure 6.0: Blood sample syringes sent to WPI for Cell Counts

Results and Discussion

Although this experiment was done on smaller scale than last year's experiment done by Lapointe & Hinkley (MQP, 2006), the results gathered were significant. In the beginning of the study 34 horseshoe crabs were collected. After escapes and pre-study mortality, a total of 22 crabs were left and sampled each week. In order to prevent the further loss of crabs, we found that adjusting the cage and frame of the PVC piping, and by hammering the fencing deep enough into the marsh the subjects were unable to dig under it.

In Figure 7.0 below, the total cells/mL of the control group (unbled) are shown from 06/01/2006 to 07/07/2006. This data indicates that the control crabs varied as much as the experimental group (bled) and that most of the crabs' blood cell counts peaked in the middle of the summer. The only exception was the crab 'RR1'. The decreasing blood cell levels in total cells/mL suggest that the crab was moribund throughout the experiment.

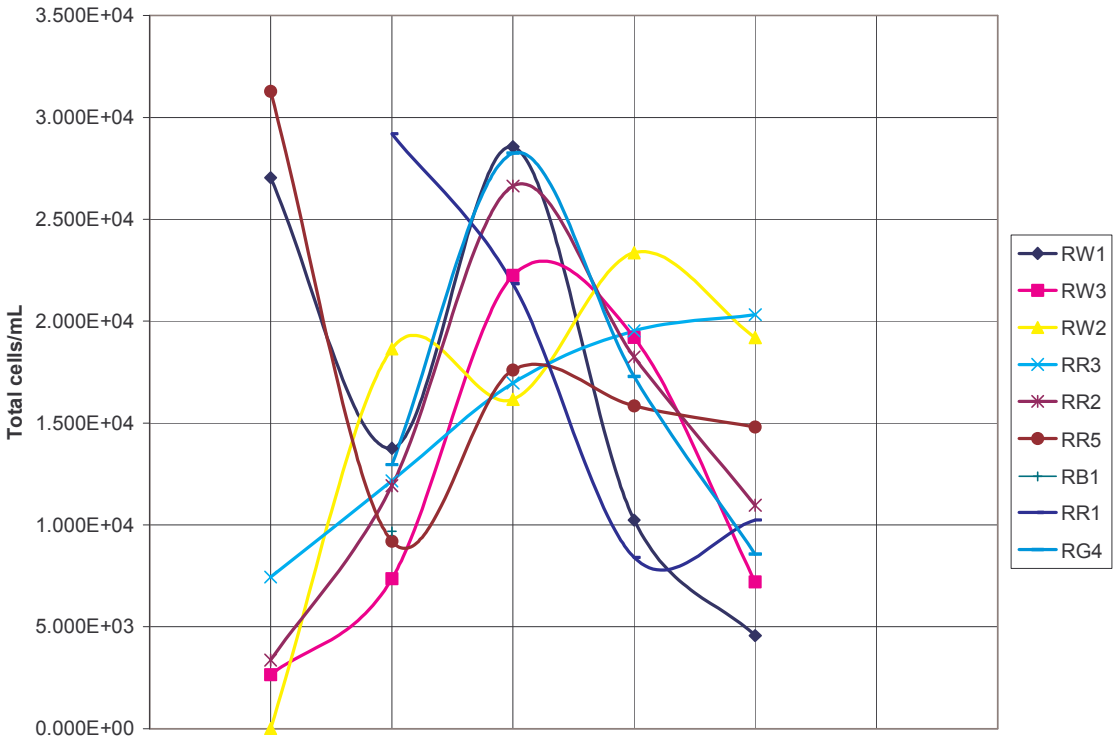

Figure 7.0: Total cells/mL from 06/01/2006 – 07/06/2006 of Control Crabs.

Figure 8.0 shows the total cells/mL of the experiment group (bled) from 06/01/2006 to 07/07/2006. Our data indicates that the majority of the bled crabs rebounded to higher levels of total cells/mL after the bleed. Crab 'LW5' shows an dramatic increase in total cells/mL throughout the study, eventually leading to a peak at roughly 3.75 x 10^4 cells/mL. Showing even more of an impressive increase was crab 'LW1'. This crab showed an initial cell count of 5.00 x 10^3 cells/mL after the initial bleed. Twenty days later, the crab's blood cell counts were 400% the initial value, totaling 2.00 x 10^4cells/mL. The only exceptions to the experimental data were crabs LW4 and LG3, which never fully rebounded to their initial blood cell levels. Unfortunately, the holding pen was not fully escape-proof at the beginning of the study, and crabs LW2 and LG2 were lost. However, soon after the crabs had escaped, the group

assessed the problem and was able to prevent further escapes by maintaining the pen as mentioned earlier in the results section.

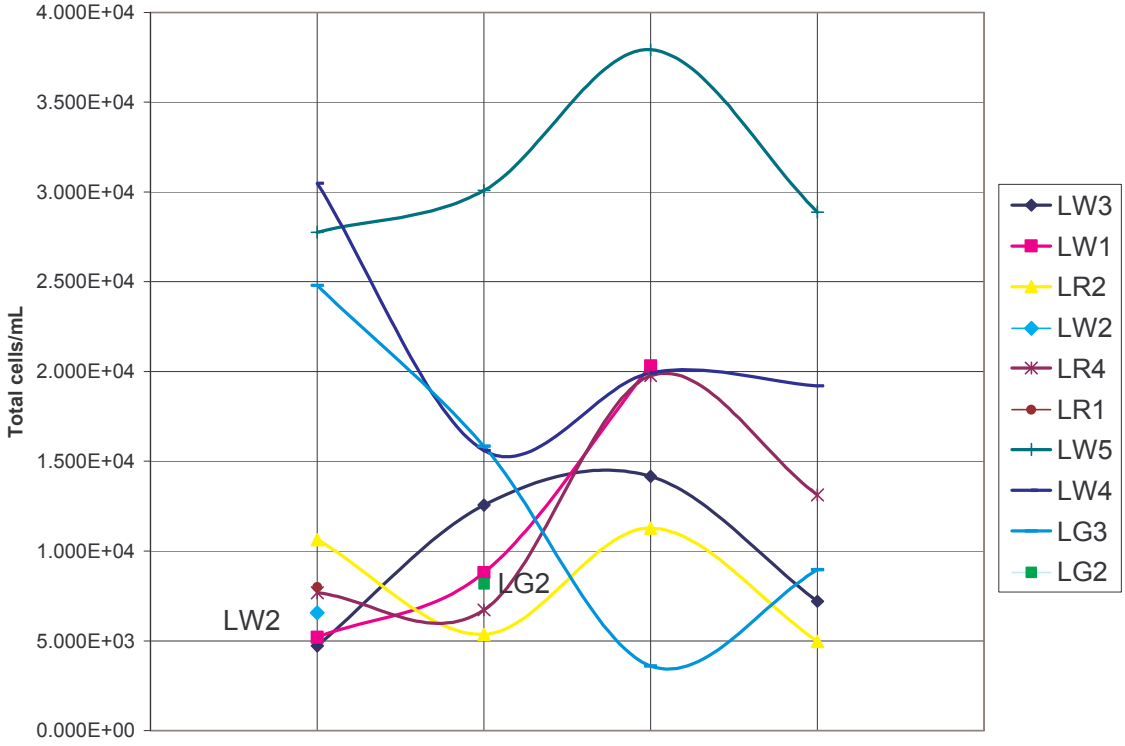

Figure 8.0: Total cells/mL from 06/01/2006 – 07/06/2006 of Experimental Crabs. (Note, LW2 and LG2 escaped after initial sample)

In addition to the total cells/mL calculated in the above figures, a 95% confidence interval was calculated using the means of both groups *(bled and unbled)* using ± 2 standard error limits. The data seen in Figure 9.0 shows that the mean total cells/mL between the two groups were within range of each other and that there is no significant difference between the experimental crabs and the control crabs. On 07/03/2006 samples were taken from the control group to use an initial reading for a second bleed which took place on 07/05/2006. The values calculated were used to determine the cell-count depressions that occur post-bleed. Figure 10.0 is an analysis of the 95% confidence interval of the second bleed showing this slight depression in 6 out of the 7 crabs

sampled. This illustrates the ability of the horseshoe crab to uptake seawater in order to replenish blood volume. The absorbed seawater used to restore the loss of blood plasma in the bled crabs dilutes the blood volume, leaving the crab with a lower total cells/mL.

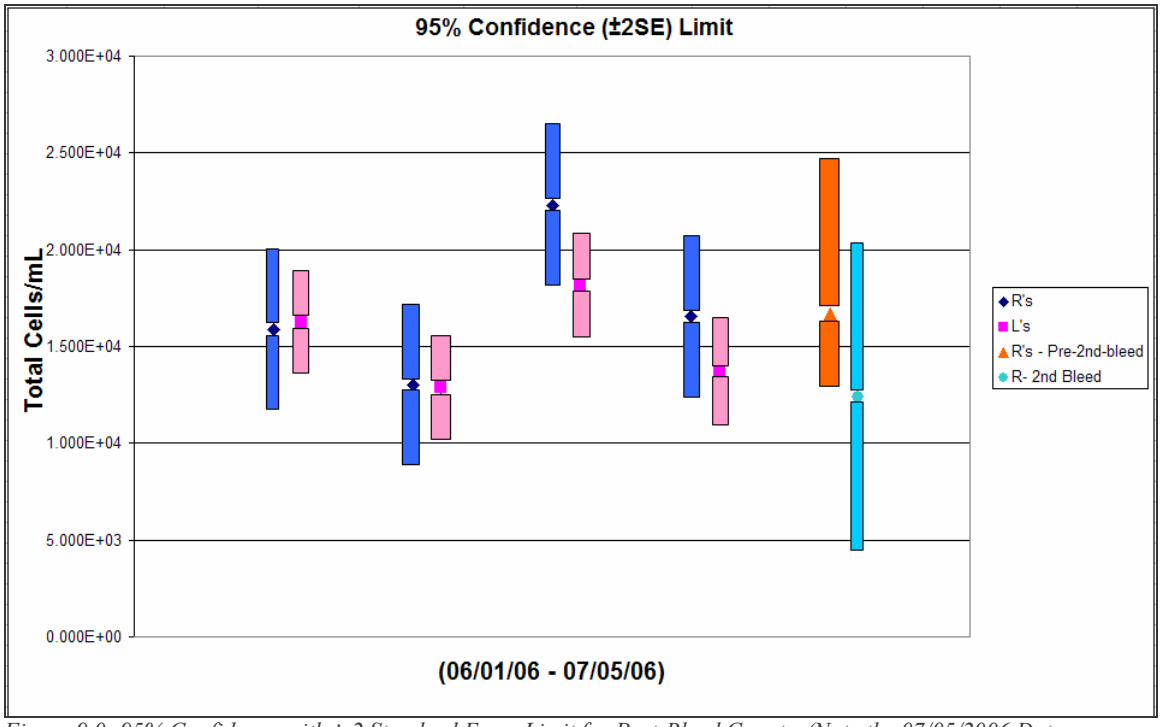

Figure 9.0: 95% Confidence with ± 2 Standard Error Limit for Post-Bleed Counts. (Note the 07/05/2006 Data represents a 2nd bleed performed on Control Group)

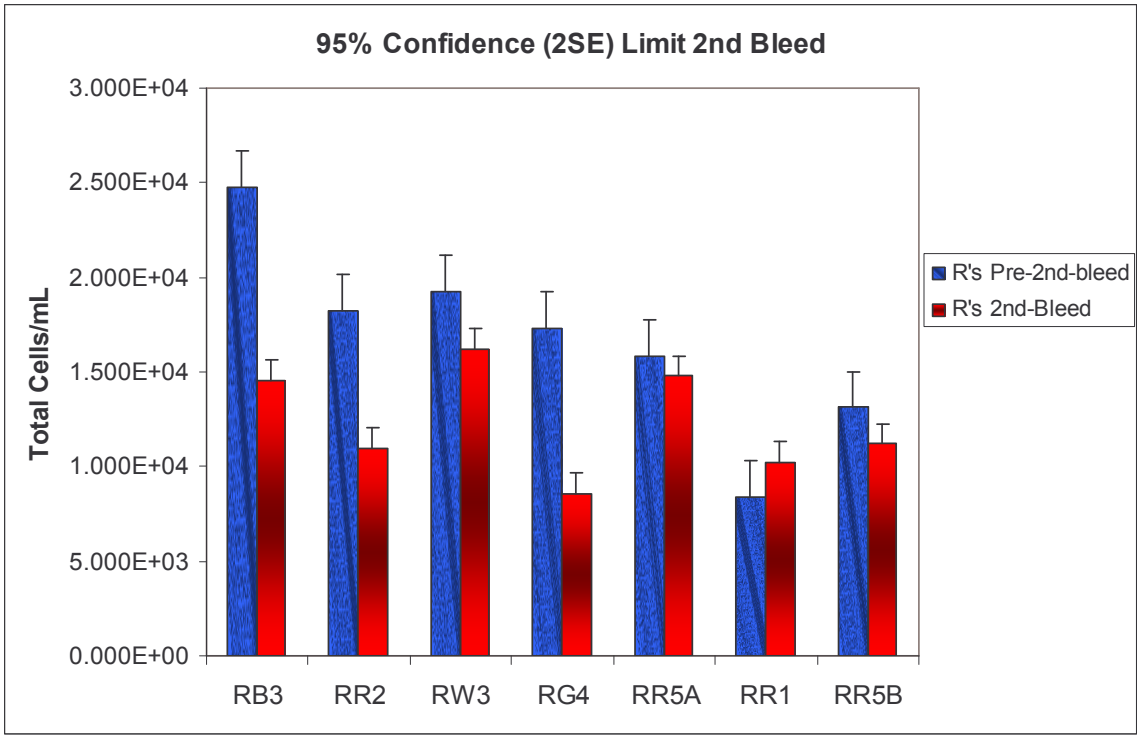

Figure 10.0: 95% Confidence with + 2 Standard Error Limit for 07/05/2006 Post-Bleed Counts.

After some initial escapes from the pen, we found that by tapping the frame of the PVC pipe into the marsh it prevented the crabs from digging under it. This routine maintenance proved to be an effective way of creating an escape-proof "natural habitat". Our data shows an 11.1% mortality rate in the control crabs as opposed to a 20% mortality rate in the exsanguinated crabs. The small sample size precludes statistical analysis of mortality rates. Cell count rebounding indicates that the industry practice of bleeding once per season is conservative. A second bleed may be possible based on the data above.

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Appendix A

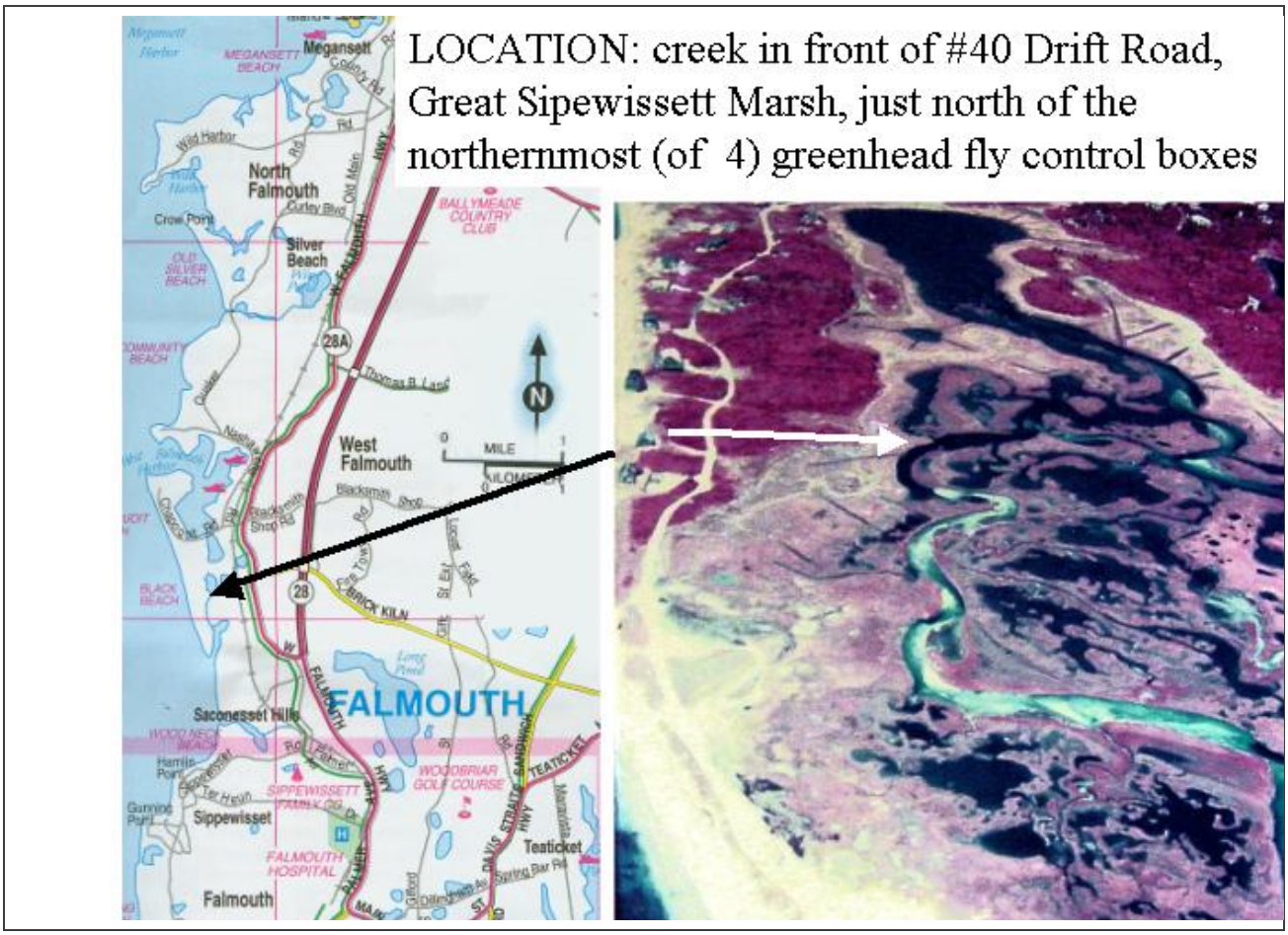
Location:

Opposite #40 Drift Road, just north of the northernmost (of 4) greenhead fly control boxes. False-color infrared aerial photo: trees dark red, marsh plants pink, mud bottom black, sand bottom white.

:

Site approval has been given by: Shellfish Warden R. Charles Martinsen III (508 457 2536),

- Falmouth Department of Natural Resources (Mark Patton, Director),
- Conservation Agent Mark Kasprzyk: 508-495-5447,
- Virginia T. Kuykendal, owner of 40 Drift Road PO Box 213 Upton MA 01568-0213; 508-529 6615,
- awaiting approval of Frank Germano, Division of Marine Fisheries, 508-910-6344



Appendix A: Location of Horseshoe Crab Holding Pen (Lapointe & Hinkley MQP, 2006)

Appendix B

Raw Data:

6/1/2006				Richard Biagiotti	Michael Bruninghaus	
				total cells/mL = ((# counted/0.1ul)/ ((5/16)(1mm²)) * 5		Average total cells/mL
	Richard Biagiotti	Michael Bruninghaus				
RW1	169	169		2.704E+04	2.704E+04	2.704E+04
LW3	26	33		4.160E+03	5.280E+03	4.720E+03
LR3	231	227		3.696E+04	3.632E+04	3.664E+04
RW3	18	15		2.880E+03	2.400E+03	2.640E+03
LW1	32	33		5.120E+03	5.280E+03	5.200E+03
RW2	116	117		1.856E+04	1.872E+04	1.864E+04
RR3	47	46		7.520E+03	7.360E+03	7.440E+03
LR2	67	66		1.072E+04	1.056E+04	1.064E+04
LW2	42	40		6.720E+03	6.400E+03	6.560E+03
RR2	19	23		3.040E+03	3.680E+03	3.360E+03
LR4	34	62		5.440E+03	9.920E+03	7.680E+03
LR1	41	59		6.560E+03	9.440E+03	8.000E+03
LW5	169	178		2.704E+04	2.848E+04	2.776E+04
LW4	167	214		2.672E+04	3.424E+04	3.048E+04
RR5	174	217		2.784E+04	3.472E+04	3.128E+04
LG3	149	161		2.384E+04	2.576E+04	2.480E+04
UNK1	326	311		5.216E+04	4.976E+04	5.096E+04
RW5	125	133		2.000E+04	2.128E+04	2.064E+04

6/11/2006						
	Richard Biagiotti	***Michael Bruninghaus***				
RB1	62	59		9.920E+03	9.440E+03	9.680E+03
RR1	153	212		2.448E+04	3.392E+04	2.920E+04
RR2	70	79		1.120E+04	1.264E+04	1.192E+04
RW1	86	86		1.376E+04	1.376E+04	1.376E+04
RW2	114	88		1.824E+04	1.408E+04	1.616E+04
RW3	49	43		7.840E+03	6.880E+03	7.360E+03
RG4	83	79		1.328E+04	1.264E+04	1.296E+04
RW5	68	74		1.088E+04	1.184E+04	1.136E+04
RR3	71	81		1.136E+04	1.296E+04	1.216E+04
RR4	27	29		4.320E+03	4.640E+03	4.480E+03
LG3	92	106		1.472E+04	1.696E+04	1.584E+04
LR2	33	34		5.280E+03	5.440E+03	5.360E+03
RR5	54	61		8.640E+03	9.760E+03	9.200E+03
LW3	75	82		1.200E+04	1.312E+04	1.256E+04
RB3	123	98		1.968E+04	1.568E+04	1.768E+04
LG2	45	57		7.200E+03	9.120E+03	8.160E+03
LW5	191	185		3.056E+04	2.960E+04	3.008E+04
LW4	87	108		1.392E+04	1.728E+04	1.560E+04
LR4	31	53		4.960E+03	8.480E+03	6.720E+03
LW1	57	53		9.120E+03	8.480E+03	8.800E+03

6/24/2006						
	Richard Biagiotti	*Michael Bruninghaus*				
LW4	128	121		2.048E+04	1.936E+04	1.992E+04
RW5	78	91		1.248E+04	1.456E+04	1.352E+04
RR5	108	112		1.728E+04	1.792E+04	1.760E+04
LR2	69	72		1.104E+04	1.152E+04	1.128E+04
LR4	119	128		1.904E+04	2.048E+04	1.976E+04
RG4	144	209		2.304E+04	3.344E+04	2.824E+04
LW3	86	91		1.376E+04	1.456E+04	1.416E+04
RB3	119	179		1.904E+04	2.864E+04	2.384E+04
RR2	143	190		2.288E+04	3.040E+04	2.664E+04
LG3	22	23		3.520E+03	3.680E+03	3.600E+03
RW2	154	138		2.464E+04	2.208E+04	2.336E+04
RW3	153	125		2.448E+04	2.000E+04	2.224E+04
LW1	136	118		2.176E+04	1.888E+04	2.032E+04
LW5	235	239		3.760E+04	3.824E+04	3.792E+04
RR3	106	106		1.696E+04	1.696E+04	1.696E+04
RR1	138	135		2.208E+04	2.160E+04	2.184E+04
RW1	176	181		2.816E+04	2.896E+04	2.856E+04

7/2/2006						
	Richard Biagiotti	*Michael Bruninghaus*				
RR2	120	108		1.920E+04	1.728E+04	1.824E+04
RW3	114	126		1.824E+04	2.016E+04	1.920E+04
LG3	50	62		8.000E+03	9.920E+03	8.960E+03
LW5	167	194		2.672E+04	3.104E+04	2.888E+04
LR2	22	40		3.520E+03	6.400E+03	4.960E+03
RR4	114	91		1.824E+04	1.456E+04	1.640E+04
RW2	114	126		1.824E+04	2.016E+04	1.920E+04
LW4	131	109		2.096E+04	1.744E+04	1.920E+04
RG4	111	105		1.776E+04	1.680E+04	1.728E+04
RR1	54	51		8.640E+03	8.160E+03	8.400E+03
RR3	115	129		1.840E+04	2.064E+04	1.952E+04
RB3	160	149		2.560E+04	2.384E+04	2.472E+04
RR5	83	115		1.328E+04	1.840E+04	1.584E+04
LR4	85	79		1.360E+04	1.264E+04	1.312E+04
RR5	84	80		1.344E+04	1.280E+04	1.312E+04
LW3	44	46		7.040E+03	7.360E+03	7.200E+03
RW1	69	59		1.104E+04	9.440E+03	1.024E+04

7/5/2006 - EXSANG - 7/3/2006						
	Richard Biagiotti	*Michael Bruninghaus*				
RB3-BLED	92	90		1.472E+04	1.440E+04	1.456E+04
RW1	27	30		4.320E+03	4.800E+03	4.560E+03
RR2-BLED	72	65		1.152E+04	1.040E+04	1.096E+04
RW3-BLED	113	90		1.808E+04	1.440E+04	1.624E+04
RR3	129	125		2.064E+04	2.000E+04	2.032E+04
RG4-BLED	47	60		7.520E+03	9.600E+03	8.560E+03
RW3	38	52		6.080E+03	8.320E+03	7.200E+03
RR5-BLED	91	94		1.456E+04	1.504E+04	1.480E+04
RR1-BLED	75	53		1.200E+04	8.480E+03	1.024E+04
RR4	49	62		7.840E+03	9.920E+03	8.880E+03
RR5B-BLED	65	75		1.040E+04	1.200E+04	1.120E+04